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Vps28 implicated in α-Synuclein Pathobiology: Building Support for Lysosome-mediated Degradation

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Summary

Misfolding and toxic accumulation of _-synuclein is thought to cause Parkinson's disease. Developing ways to reduce α-Synuclein accumulation may lead to future treatments. The dominant model of _-synuclein degradation is by the proteasome degradation pathway where _-synuclein toxicity has been associated with malfunctions in this pathway. However, evidence is building to suggest that the multivesicular body (MVB) sorting pathway to degradation via the vacuole/ lysosome also degrades _-synuclein and that malfunctions in this pathway lead to _-synuclein accumulation and toxicity. Here we demonstrate that the absence of vps28, an essential component of the MVB sorting pathway, increases the toxicity of both wild type and mutant forms of _-synuclein. The absence of vps28 also significantly alters the cellular distribution and localization of _-synuclein and increases its cytoplasmic aggregation. We propose that dysfunction of the MVB sorting pathway to the vacuole/lysosome can lead to the pathology linked to Parkinson's disease, including cell death.

Introduction

Parkinson's Disease (PD), the most common neurodegenerative disorder, is physically characterized by slowed movement, resting tremor, muscular rigidity, and postural instability (Giasson and Lee, 2003). One defining quantitative feature of PD is the death of dopaminergic neurons in the substantia nigra, which normally function to produce the neurotransmitter dopamine that is responsible for smooth, coordinated muscle movement (Forno, 1996). Within dying neurons are cytoplasmic inclusions of misfolded _-synuclein called Lewy bodies and the associated abnormal neurons called Lewy neurites in some of the remaining neurons of the same region (Goedert, 2001; Vekrellis et al., 2004; Spillantini et al., 1998).

_-Synuclein is a cytoplasmic, membrane associated protein of 140 amino acids abundant at presynaptic terminals in the nervous system (Goedert, 1997; Davidson et al., 1998; McLean et al., 2000). While the precise function of _-synuclein remains unknown, it has been implicated in synaptic remodeling and plasticity (Kahl et al., 2000). The three missense point mutations of the _-synuclein gene on chromosome 4, A30P, A53T and E46K, associated with familial PD may result in the misfolding and fibril formation of the _-synuclein protein and its subsequent aggregation into Lewy bodies (Goedert, 2001; Nussbaum and Polymeropoulos, 1997; Kruger et al., 1998; Conway, 1998; Zarranz, 2004). Increasingly, evidence shows that abnormalities in the degradation of misfolded _-synuclein underlie the toxic affects seen in PD but the precise nature of this degradation remains uncharacterized (McNaught et al., 2001).

One avenue of possible treatment for PD focuses on clearing these misfolded proteins from cells. Genetic evidence shows that _-synuclein is degraded by the ubiquitin-proteasome degradation pathway (Bennet et al., 1999; Holtz and O'Malley, 2003). Under normal circumstances, misfolded proteins, including misfolded _-synuclein, are targeted to the proteasomal degradation pathway by polyubiquitination (Thrower et al., 2000). Also, several genetic mutations associated with PD inhibit elements of the ubiquitin-proteasome degradation pathway including mutations in Parkin on chromosome 6, which normally functions as an ubiquitin ligase, PARK 3 on chromosome 2, and in ubiquitin C-terminal hydrolase L1 (Ceichanover and Brundin 2003; McNaught et al., 2002). This evidence points to the ubiquitin-proteasome degradation pathway as the mechanism for _-synuclein degradation and that malfunctions in this pathway play a major role in PD.

However, pharmacological studies indicate that proteasome inhibitors do not alter cellular levels of _-synuclein or Parkin (Rideout and Stefanis, 2002; Biasini et al., 2004). In fact, _-synuclein aggregation has been implicated as a causative agent in dysfunction of the ubiquitin-proteasome system as apposed to dysfunction in the system causing _-synuclein aggregation (Snyder et al., 2003). Therefore, a system other than the ubiquitin-proteasome degradation pathway is implicated in the breakdown of _-synuclein—the vacuole/lysosome pathway.

The vacuole/lysosome has its own system of targeting and transport and typically degrades extracellular particles, worn out organelles, and membrane proteins, a category including _-synuclein. Ubiquitination, in this case monoubiquitination, has been shown to sufficiently target proteins to endocytic degradation pathways, such as the multivesicular body (MVB) sorting pathway, to the vacuole/lysosome (Hochstrasser, 1996; Katzmann et al., 2001). Recently, in mammalian cells, some _-synuclein translocates to lysosomes for degradation and lysosomal inhibitors have been shown to increase the intracellular levels of _-synuclein (Cuervo et al., 2004; Lee et al., 2004).

The MVB pathway is composed by a number of essential vacuolar protein sorting proteins (vps proteins) required (Odorizzi et al., 1998) which either function alone or as part of a complex. One such complex is the Endosomal Sorting Complex Required for Transport 1 (ESCRT 1), a 350 kDa complex composed of vps23, vps28, and vps37 (Katzmann et al., 2001). The specific role of vps28 (human ortholog hvps28) has yet to be characterized, though it has been implicated as necessary for vps23 function (Bishop and Woodman, 2001), the ESCRT 1 component that binds Ub (Katzmann et al., 2001; Babst et al., 2000). We propose that _-synuclein is targeted to the MVB sorting pathway for degradation by the vacuole/lysosome and vps28 is a required element for the functioning of this

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pathway. Therefore, it can be conjectured that the absence of a functioning vps28 gene would lead to disruptions in the MVB sorting pathway and therefore degradation by the vacuole/lysosome. The disruption of degradation would lead to an accumulation of proteins, including _-synuclein, which is linked to _-synuclein toxicity and PD symptoms.

To gain understanding of the role of vps28 in _-synuclein degradation and toxicity, a number of *in vivo* experiments were performed using a *S. cerevisiae* PD model developed in our lab (Johnson et al., 2003; Sharma et al., 2004) as yeast has already proven to be a useful model for studying neurodegenerative diseases, including Alzheimer's and Huntington's Disease in addition to PD (Outeiro and Muchowski, 2004; Outeiro and Lindquist, 2003). In vps28 deficient *S. cerevisiae* mutants, wild type _-synuclein toxicity has been shown to be greater than in cells with an intact genome (Willingham et al., 2003). We assessed the toxicity of wild type and mutant _-synucleins (A30P, A53T, and A30P/A53T) through growth curve analysis and dilution series spotting of strains both with and without vps28. The cellular distribution and accumulation of wild type and mutant _-synucleins in these strains were assessed through GFP microscopy and biochemical assessment was used to examine wild type _-synuclein's expression, accumulation, and stability *in vivo* over time. Together these assays construct a clearer model of the role of vps28 in α-synuclein degradation and toxicity.

Results

_- synuclein is expressed in v*ps28* strains

Vps28 was previously identified by a genetic screen to enhance the toxicity of wild type _-synuclein in *S. cerevisiae* (Willingham, et al., 2003). In order to further examine the affects of _-synuclein in vitro, *S. cerevisiae* parent wild type and *vps28* strains were transformed with wild type _-synuclein, A30P _-synuclein, A53T α-synuclein, and A30P/A53T _-synuclein tagged with GFP (green fluorescence protein). Control transformants with empty pYES2 parent plasmid and GFP alone were also created. Galactose-induced _-synuclein expression was verified via western blotting (Figure 1a). No _-synuclein expression was seen in cells transformed with the empty pYES2 parent vector _-synuclein (Figure 1a, lanes 1-2) nor cells containing the wild type _-synuclein gene grown in non-inducing glucose media (Figure 1a, lanes 5-6). For all _-synuclein transformants a single band was detected at approximately 58 kDa, corresponding to the monomeric form of _-synuclein tagged with GFP, when expression was induced by galactose (Figure 1a, lanes 4-14). Band intensity does not remain consistent between *vps28* transformants and their parent strain counterparts for mutant _-synucleins A30P, A53T, and A30P/A53T. However, these differences are not accounted for by variation in protein quantity as visualized by coomassie staining (Figure 1b). For A30P, and A30P/A53T _-synuclein mutants, band intensity was weaker for *vps28* transformants though more protein was present in the sample. This indicates that the absence of vps28 has a mild but consistent affect on mutant _-synuclein expression.

***Vps28* modestly increases α-synuclein toxicity**

To asses the impact of the lack of vps28 on growth of _-synuclein expressing cells, parent strain and *vps28* comparative growth curves were established for each construct (Figure 2a). For pYES2 parent plasmid and

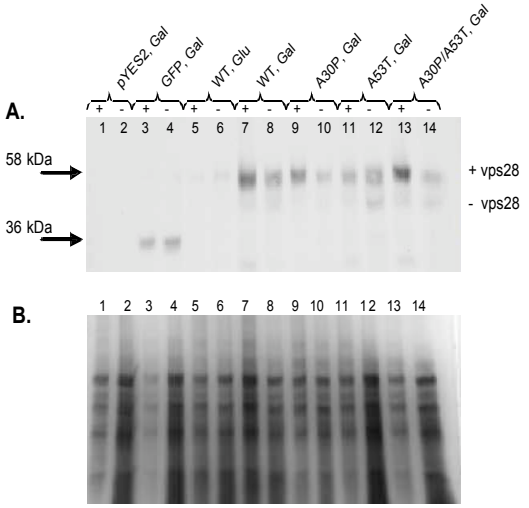

Figure 1. _-Synuclein Expression in *Vps28*
(A) *Western blotting.* All parent strain and *vps28* _-synuclein transformants showed a band at approximately 58kDa corresponding to _-synuclein expression when induced by galactose (lanes 7-14). No expression was seen in glucose grown _-synuclein transformants (lanes 5-6) nor cells containing only the pYES2 parent vector (lanes 1-2). GFP transformants produced a band at approximately 36kDa which corresponds to GFP expression when induced by galactose (lane 4). (B) *Coomassie Staining.* All lanes exhibit the same number of bands at comparable intensities indicating that the same amount of protein was loaded for each sample. Variances in intensity do not correlate with the amount of _-synuclein expression indicated by band intensity on the western blot.

GFP transformants, growth of *vps28* and parent strains was equivalent, with *vps28* exhibiting only slightly slowed growth in the pYES2 transformant.All *vps28* α-synuclein (both wild type and mutant forms) transformants exhibited slowed growth compared to parent strain when _-synuclein expression was induced. The most significant slowed growth was present in *vps28* wild type _-synuclein transformants. To further examine _-synuclein toxicity, dilution series spotting of parent strain and *vps28* transformants was plated on inducing and non-inducing media (Figure 2b). In the absence of induction, the growth of all parent strain and *vps28* transformant pairs was comparable. When _-synuclein expression was induced, the wild type *vps28* transformant exhibited notably less growth than the wild type parent strain transformant. Only a marginal difference in growth was seen in *vps28* A30P, A53T, and A30P/A53T _-synuclein transformants, when compared to their parent strain counterparts. No difference was seen between the growth of parent strain and *vps28* pYES2 or GFP transformants. Together, growth curve and spotting data indicate that the absence of the vps28 gene definitively increases wild type _-synuclein toxicity in *S. cerevisiae* while mutant _-synuclein is only marginally more toxic.

***Vps28* alters _-synuclein localization and increases peri-vacoular aggregation**

To analyze the localization of _-synuclein, _-synuclein-GFP fusion transformants were visualized through fluorescence microscopy (Figure 3). In wild type and A53T _-synuclein parent strain cells, the _-synuclein-GFP fusion localizes at the plasma membrane which is consistent with _-synuclein's characterization as a membrane associated protein and previous microscopy

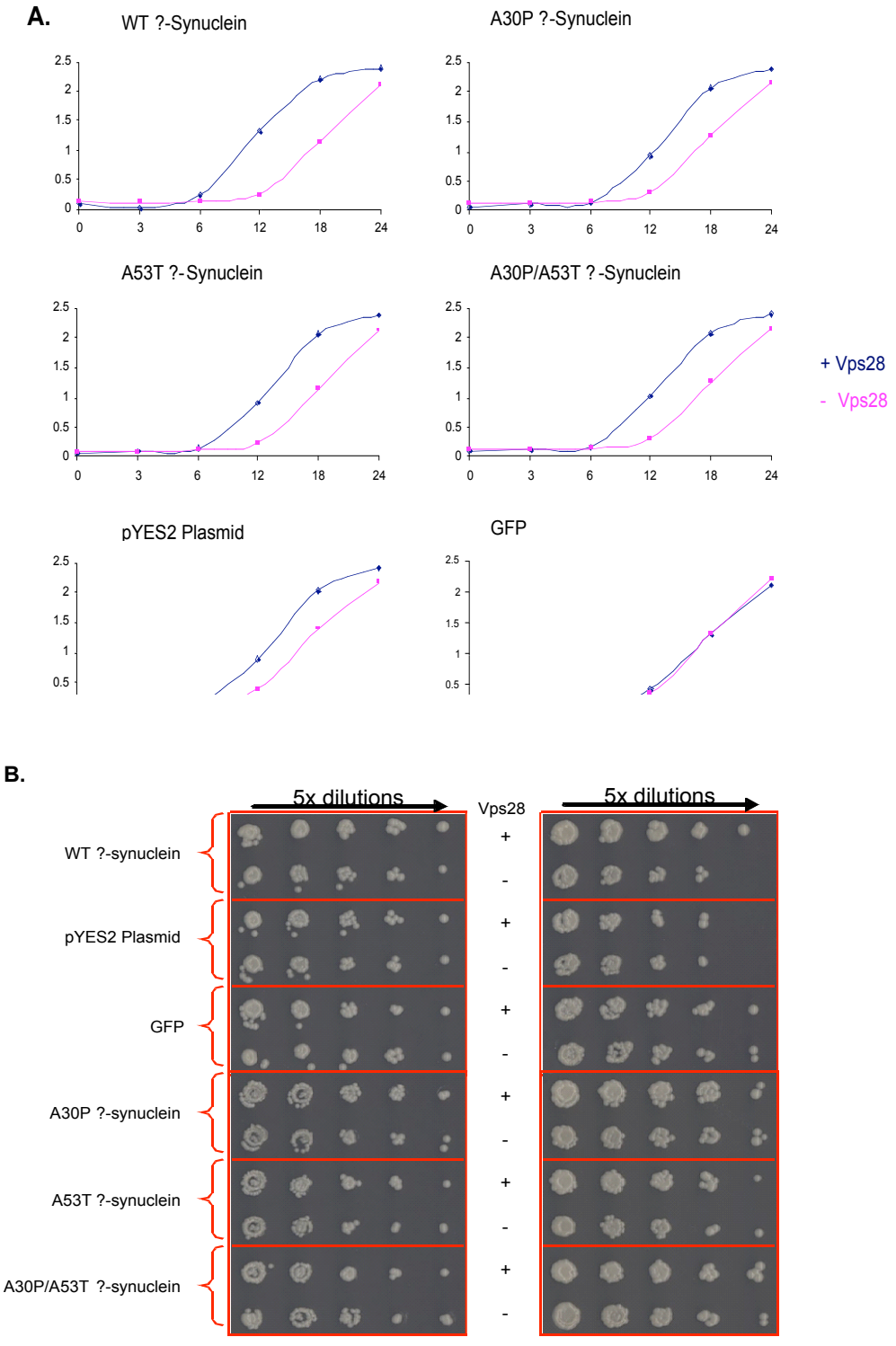

Figure 2. _-Synuclein Toxicity Assessment in *Vps28*
(A) *Growth curve.* Comparative growth curves were established using OD600 for transformants with an intact vps28 gene and those without vps28. In the absence of _-synuclein expression, both strains showed comparable growth while _-synuclein expression resulted in slightly inhibited growth of the *vps28* transformants with wild type _-synuclein exhibiting the most significant difference. **(B)** *Serial spotting on plates.* Growth was also analyzed by five fold serial dilution spotting of transformed yeast cells on non-inducing (glucose) or inducing (galactose) SC-ura plates. In the absence of _-synuclein expression, growth of all transformants was comperable. However, _-synuclein expression caused minor toxic effects in the wild type *vps28* transformant when compared to the wild type parent strain transformant. Little or no difference in growth was seen between other parent strain and *vps28* transformants when _-synuclein expression was induced.

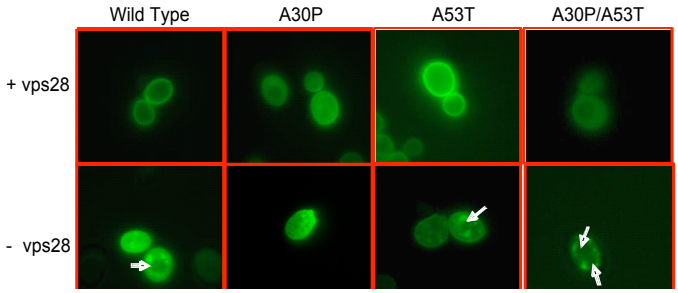

Figure 3. _-Synuclein Cellular Distribution and Accumulation in *Vps28*
After 24 hours of induction, _-synuclein-GFP exhibited peripheral localization in parent strain wild type and A53T _-synuclein transformants while _-synuclein-GFP showed cytoplasmic distribution in A30P and A30P/A53T _-synuclein transformants, as previously shown. At the same time point, *vps28* wild type _-synuclein transformant exhibited _-synuclein distribution throughout the cytoplasm, foci of accumulation, and exclusion from the vacuole. While the A30P _-synuclein *vps28* transformant remained distributed throughout the cytoplasm, foci of cytoplasmic accumulation were also seen. Both A53T and A30P/A53T _-synuclein transformants exhibited _-synuclein distribution throughout the cytoplasm and distinct foci of accumulation. All *vps28* transformants exhibited markedly different _-synuclein distribution and an increase in accumulation when compared to their parent strain counterparts.

findings (Davidson, et al., 1998; Outeiro and Lindquist, 2003). Also consistent with the same previous model, A30P _-synuclein parent strain cells exhibited _-synuclein-GFP dispersion throughout the cytoplasm, reflecting A30P _-synuclein's poor membrane binding ability (Outeiro and Lindquist, 2003). This decreased affinity for membrane binding also leads to the cytosolic distribution of A30P/A53T _-synuclein-GFP in parent strain cells. Notably, cells exhibiting cytoplasmic distribution (A30P and A30P/A53T), _-synuclein-GFP was excluded from the vacuole and in all transformants no cytoplasmic inclusions are seen.

In wild type and A53T *vps28* cells, _-synuclein-GFP lacked the expected membrane association and exhibited cytoplasmic distribution instead. Prominent cytoplasmic inclusions, appearing as foci of fluorescence, are also present in close proximity to the vacuole (indicated by white arrows in Figure 3) from which _-synuclein-GFP remains excluded. A30P and A30P/A53T _-synuclein *vps28* cells still exhibited _-synuclein-GFP dispersion throughout the cytoplasm but _-synuclein-GFP localized into numerous cytoplasmic inclusions, again in the vicinity of the _-synuclein-GFP excluding vacuole. In the absence of the vps28 gene, all forms of _-synuclein-GFP accumulate in the cytoplasm and form distinct inclusions in the vicinity of the vacuole, which excludes _-synuclein-GFP. This localization is dramatically different from localization in the presence of the vps28 gene.

α-Synuclein turnover unchanged in *vps28*
To asses the affect of vps28 absence on the persistence and stability of _-synuclein in cells, the cellular expression of _-synuclein was halted after 24 hours by transfer from inducing galactose media to non-inducing glucose media and _-synuclein levels were monitored for the following 24 hours (Figure 4). In cells with an intact vps28 gene, _-synuclein was detected at all time points during the 24 hour time course and no consistent decrease in _-synuclein presence was seen as indicated by the presence of bands at each time point. Inconsistencies in band intensity seen for cells with vps28 (lanes for 0, 0.5, and 1 hours for example) are a result of variances in the amount of total protein present in the samples (data not shown). In *vps28* cells, there was also no significant decrease in the amount of _-synuclein present in cells up to 24 hours after the loss of induction. The slight decrease seen between 0.5 and 1 hours in *vps28* cells, which may indicate that _-synuclein is degraded in absence of vps28. No bands appeared above the 58 kDa size, corresponding to the monomeric form of α-synuclein tagged with GFP, for any of the samples.

Discussion

As _-synuclein misfolding and dysfunctions in its degradation persists as an underlying cause of PD, studying _-synuclein degradation *in vivo* is of critical import (McNaught et al., 2001). Recent studies have implicated that the vacuolar/lysosomal system of degradation in the breakdown of _-synuclein (Cuervo et al., 2004; Lee et al., 2004; Willingham, et al., 2003). One important pathway to the vacuole/lysosome, the MVB pathway, is composed of a plethora of vps proteins (Odorizzi et al., 1998). The absence of vps28, as a part of the ESCRT-I complex, has been shown to increase α-synuclein toxicity, implying that vps28 plays a role in the clearance of _-synuclein from the cell (Willingham, et. al. 2003). We provide insight into _-synuclein degradation. Specifically our data suggests that _-synuclein may utilize the vacuole/lysosome pathway in addition to the proteasome degradation pathway.

Vps28 prevents α-Synuclein from being toxic to yeast
An increase in wild type _-synuclein toxicity has previously been demonstrated in *vps28* in *vivo* (Willingham, et. al., 2003). We also demonstrate an increase in the toxicity of wild type _-synuclein in *vps28* cells though the increase in toxicity was more moderate. This could be because our cells may express _-synuclein at lower levels than those used in the Willingham et. al., 2003 study. We extended this research by examining _-synuclein toxicity in _-synuclein mutants and demonstrated that the toxicity of A30P, A53T, and A30P/A53T mutant _-synuclein was also modestly increased in the absence of vps28. It was expected that toxicity would be greater in than our experiments demonstrated, especially for _-synuclein mutants when compared to wild type. The absence variation in toxicity between wild type and mutant _-synucleins implies that the absence of vps28 is responsible for toxicity exclusively and not mutations in _-synuclein itself. This explains the sporadic occurrence of PD in patients that do not have _-synuclein mutations, tying sporadic PD to the accumulation of _-synuclein due to dysfunctions in the vacuolar/lysosomal degradation pathway.

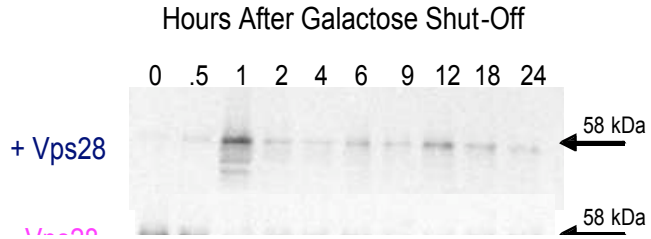

Figure 4. Biochemical assessment of wild type _-synuclein accumulation in *vps28*
Over a course of 24 hours after the halting of wild type a-synuclein synthesis, the *in vivo* levels of a-synuclein did not decline in the parent strain nor *vps28*. There was no apparent difference between the levels of wild type a-synuclein in parent strain cells and *vps28* cells throughout the time course. This indicates that the presence of vps28 does not have a discernable affect on the clearance of a-synuclein from cells 24 hours after a-synuclein expression is stopped.

α-Synuclein may be targeted to the MVB pathway
The finding that the absence of vps28 significantly alters the localization of all _-synuclein forms and increases the amount of _-synuclein cytoplasmic inclusion allowed us key insight into the role of vps28 in _-synuclein degradation. The accumulation of _-synuclein into Lewy bodies within cells has been previously linked to cell death via the disruption of a variety of cellular processes (Goedert, 2001; Vekrellis et al., 2004; Spillantini et al., 1998). The presence of cytoplasmic inclusions of all forms of _-synuclein in *vps28* cells implies that the absence of vps28 leads to the accumulation of _-synuclein within the cell, a key aspect of PD. This dramatic difference seen in the localization of all forms of _-synuclein between cells with the vps28 gene and cells without indicates that vps28 is key in the removal of _-synucleins from the cell. As an integral part of the MVB sorting pathway to the lysosome, the affect of vps28 on _-synuclein behavior points to the importance of the MVB pathway and the lysosome in _-synuclein degradation.

Vps28 presence does not discernibly alter _-synuclein clearance
The presence of vps28 did not have discernable affect on the presence of wild type _-synuclein up to 24 hours after the _-synuclein expression was halted. The persistence of wild type _-synuclein in cells of both parent strain and *vps28* may be due to the fact that _-synuclein may be present in SDS-soluble aggregates which broke down to monomers in sample buffer in both parent and *vps28* strains. This would explain the consistency of band size at 58 kDa. Also, alpha synuclein is a reasonably stable protein in yeast and the lack of vps28 may not be enough to increase this stability by a large, discernable amount. Therefore, the rate of disappearance from yeast cytosol would appear similar in cells with and without vps28. *Vps28* may impact wild type _-synuclein stability, but its effects may not be dramatic enough to capture in this assay.

Role of Vps28 in _-synuclein Biology
In this study we demonstrate that vps28, as an essential part of the MVB sorting pathway to the vacuole/lysosome, plays an integral role in the clearance of all forms of _-synuclein from the cell and that cells lacking vps28 experience an increase in _-synuclein accumulation within the cell. This accumulation, we propose, explains the toxic affects of _ -synuclein.

The misfolding of _-synuclein and the subsequent formation of Lewy bodies has been linked to cell death in PD (Goedert, 2001; Vekrellis et al., 2004; Spillantini et al., 1998). The ubiquitin-proteasome degradation pathway has been shown to degrade misfolded _-synuclein (Bennet et al., 1999; Holtz and O'Malley, 2003) and dysfunction of this pathway has been linked to _-synuclein accumulation and aggregation (Sharma, 2004). However, the function of the ubiquitin-proteasome in clearing _-synuclein from the cell has been brought into question, implicating an alternate method of _-synuclein degradation (Rideout and Stefanis, 2002; Biasini et al., 2004). Conveniently, _-synuclein has also been shown to be targeted to and degraded by the vacuole/lysosome (Cuervo et al., 2004; Lee et al., 2004).

Essential to the degradation of proteins by the vacuole/lysosome is the MVB sorting pathway, of which vps28 is a component (Katzmann et al., 2001). Our data shows that disruption of this pathway, via the absence of the vps28, elevates the toxicity of all forms of _-synuclein in addition to increasing its accumulation and aggregation within cells. This indicates that disruption of the MVB sorting pathway and therefore transport to the vacuole/lysosome for degradation has similar affects as the disruption of the ubiquitin-proteasome degradation pathway (Snyder et al., 2003, McNaught, et. al., 2003). Therefore, we propose a model of _-synuclein degradation in which it can be targeted to either the proteasome, targeted by polyubiquitination (Thrower et al., 2000), or the vacuole/lysosome, targeted by monoubiquitination (Hochstrasser, 1996; Katzmann et al., 2001), for degradation depending on its ubiquitination state (Figure 5). It is likely that these pathways work in conjunction to clear _-synuclein from the cell and that disruption of either of these pathways can cause accumulation of _-synuclein, disruption of cellular processes, and eventually cell death as seen in PD.

Future research could be conducted to understand the role of other vps proteins in _-synuclein clearance from the cell through the vacuole/lysosome degradation pathway to understand how regulation of vesicular transport could be used to treat PD. Also, confirmation of the ubiquitination state of _-synuclein in *vps28* would point to how _-synuclein is selected for degradation by the two main degradation machineries, the proteasome or the lysosome.

Experimental Procedures

Strains and Transformation
Human wild type and A53T mutant _-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and A30P/A53T mutant _-synuclein were created using site directed mutagensis (Invitrogen) from wild type and A53T

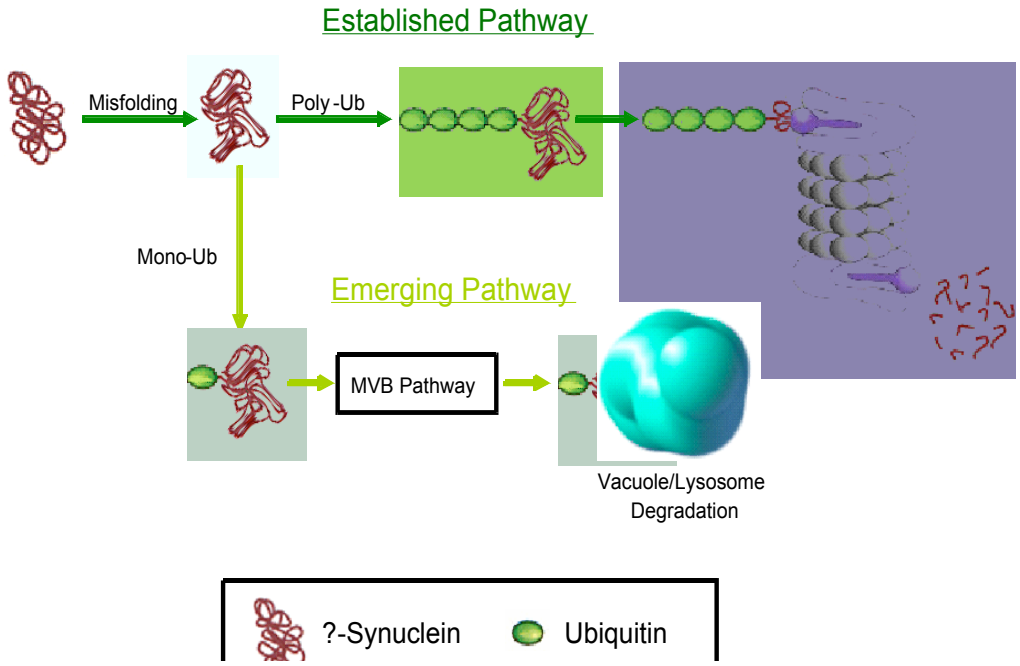

Figure 5. Model for the effects of *vps28* on _-synuclein toxicity
Previous research has established that misfolded _-synuclein is polyubiquitinated and degraded by the ubiquitin-proteasome system. Emerging evidence indicates that _-synuclein is also degraded by the vacuole/lysosome, with monoubiquitination being the possible targeting mechanism for this pathway. Together, both of these pathways could operate in conjunction to clear misfolded _-synuclein from the cell Dysfunction in either of these pathways could disrupt this clearance and lead to _-synuclein accumulation and subsequent cell death associated with PD.

mutant _-synuclein, respectively. Wild type and mutant _-synuclein cDNAs were subcloned into the pcDNA3.1 expression vector, which were then amplified and subcloned into the pYES2.1/V5-His-Topo yeast expression vector (Invitrogen). α-Synuclein expression plasmids were transformed as described (Burke, 2000) into URA-3 deficient *S. cerevisiae* 4741 and *vps28* strains. For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-ura). Presence of α-synuclein constructs was confirmed by polymerase chain reaction. The pYES2.1 vector, containing a galactose inducible promoter (GAL1), allowed for regulated _-synuclein expression. Table I lists the transformed yeast strains used in this study.

Western Blotting
Yeast cells ($2.5x10^7$ cells/ml) were washed in 50mM Tris (pH 7.5), 10mM Nan_3 and solubilized Electrophoresis Sample Buffer (ESB, Burke, 2000) containing 2% SDS, 80mM Tris (pH 6.8), 10% glycerol, 1.5% DTT, 1mg.ml bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton-X 100, 1mM PMSF, 1mM benzamidine, 1mM sodium orthovanadate, 0.7_g/ml pepstatin A, 0.5_g/ml leupeptin, 10_g/ml E64, 2_g/ml aprotinin, and 2_g/ml chymostatin). Samples were run on pre-cast 10-20% Tris-Glycine SDS gels (Invitrogen), using SeeBlue as the molecular standard (Invitrogen). Gels were transferred to PVDF membranes and probed with anti-V5 antibody (Invitrogen).

Growth Analysis of Toxicity
For OD_{600} analysis, absorbance readings of yeast cells expressing a-synuclein were taken at 600nm using a Hitachi U-200 spectrophotometer at 0, 3, 6, 12, 18, 24, and 36 hours after induction. For spotting, yeast cells were grown in SC-ura+glucose to mid-log phase, washed with H_2O, and then resuspended in SC-ura+raffinose for 4 hours. Cell densities were normalized and a dilution series of 6, 1:10 serial dilutions were made for each sample. These dilution series were spotted on SC-ura+galactose plates. Cells were photographed after 2 days of growth.

Loss of Induction
Yeast cells grown in SC-ura+glucose media overnight were washed in H_2O and resuspended in SC-ura+galactose media to induce α-synuclein expression. After 18 hours of induction, cells were transferred back to SC-ura+glucose media, halting the further synthesis of α-synuclein. At 0, .5, 1, 2, 4, 6, 9, 12, 18, and 24 hours after the shut off of α-synuclein synthesis, whole cell lysates were made from each strain, and a Western blot using α-synuclein antibody was performed.

GFP Microscopy
Vps28 yeast cells were visualized under blue-filter settings using a Zeiss Axiovert-100 fluorescent microscope 18-24 hours after galactose-induced α-synuclein expression. Images were acquired with Metamorph 4.0 imaging software. Correspondingly transformed 4741 strains were visualized as a control and used as the basis for α-synuclein distribution and accumulation when a functioning MVB sorting pathway is present.

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